

Expression of a pathogen-induced cysteine protease (*AdCP*) in tapetum results in male sterility in transgenic tobacco

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Abstract Usable male sterility systems have immense potential in developing hybrid varieties in crop plants, which can also be used as a biological safety containment to prevent horizontal transgene flow. Barnase-Barstar system developed earlier was the first approach to engineer male sterility in plants. In an analogous situation, we have evolved a system of inducing pollen abortion and male sterility in transgenic tobacco by expressing a plant gene coding for a protein with known developmental function in contrast to the Barnase-Barstar system, which deploys genes of prokaryotic origin, i.e., from *Bacillus amyloliquefaciens*. We have used a plant pathogen-induced gene, cysteine protease for inducing male sterility. This gene was identified in the wild peanut, *Arachis diogeni* differentially expressed when it was challenged with the late leaf spot pathogen, *Phaeoisariopsis personata*. *Arachis diogeni* cysteine protease (*AdCP*) was expressed under the strong tapetum-specific promoter (TA29) and tobacco transformants were generated. Morphological and histological analysis of *AdCP* transgenic plants showed ablated tapetum and complete pollen abortion in three transgenic lines. Furthermore, transcript analysis displayed the expression of cysteine protease in these male sterile lines and the expression

of the protein was identified in western blot analysis using its polyclonal antibody raised in the rabbit system.

Keywords Male sterility · Pollen abortion · Cysteine protease · *AdCP* · TA29 promoter · *Arachis diogeni*

Introduction

From the agriculture perspective, development of male sterile plants is one of the valuable tools in the seed industry for generating hybrid seeds without the need of labor-intensive hand emasculating and pollination. In plants, male sterility is often defined as failure of plants to produce functional anthers, male gametes or pollen grains. This phenomenon is commonly observed in cytoplasmic male sterility (CMS), which is a maternally inherited trait in plants (Hanson 1991). Using the CMS system with appropriate genes for fertility restoration is one of the conventional methods for hybrid seed production. The deployment of hybrid varieties is the best option for a quantum leap in plant productivity and stability over years and environments. This stability in performance is because of the hybrid vigor or heterosis in the hybrids, which results in superior performance in a heterozygous hybrid progeny over both homozygous parents with respect to traits such as growth rate, reproductive success and yield (Lippman and Zamir 2007). However, the availability of these systems without floral deformities is relatively meager with several of the important crop plants lacking usable male sterility systems. Commercial production of hybrids is feasible in such crops only if a reliable and effective pollination control system is available. Developing genetically engineered male sterile system provides a better and effective way for controlling pollination in crop plants in which suitable natural methods are not available.

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The first genetically engineered male sterile plants were developed by expressing the bacterial gene for a protein, ribonuclease (Barnase) from the bacterium, *Bacillus amyloliquefaciens* (Mariani et al. 1990) in tapetal cell layer, which supports microsporogenesis by providing nourishment to the microsporogenous tissues and developing pollen grains; any abnormality in tapetal function would have a bearing on microsporogenesis and pollen development. In addition to this, many other strategies and genes have also been tried to disrupt anther development by using an appropriate anther-specific promoter. For instance, altering metabolic process by expressing mutated glutamine synthetase (*GSI* and *GS2*) in anther (Ribarits et al. 2007), RNA silencing method by suppressing rice allene oxide synthase genes (*OsAOS1*, *OsAOS2*) (Bae et al. 2010), RNA editing as a tool by expressing unedited ATP9 in mitochondria (Hermould et al. 1998), by heterologous expression of CMS associated chimeric genes like *orf456* (Kim et al. 2007), *orf129* (Yamamoto et al. 2008), *orfH522* (Nizampatnam et al. 2009), via chloroplast engineering by expressing *phaA* gene that codes for β -ketothiolase in plastid through plastid transformation (Ruiz and Daniell 2005), developing conditional male sterile system by expressing temperature sensitive diphtheria toxin A-chain (Guerineau et al. 2003), use of *AtBECLIN1* gene (Singh et al. 2010), use of modified form D-amino acid oxidase (DAAO) (Hawkes et al. 2011), and the use of bacterial gene *argE* (Kriete et al. 1996) were successfully tried for developing engineered male sterile plants.

In plants, several reports suggest a critical regulatory role of cysteine proteases in programmed cell death (PCD, Pennell and Lamb 1997; Trobacher et al. 2006). A number of genes encoding papain-like cysteine proteases have been identified from senescing organs including leaves (Ueda et al. 2000; Gepstein et al. 2003), flowers (Eason et al. 2002), legume nodules (Kardailsky and Brewin 1996) and germinating seeds (Ling et al. 2003). Some of the cysteine proteases get activated during oxidative stress induced PCD (Solomon et al. 1999) and also during developmental PCD. Furthermore, overexpression of cysteine protease *BoCysP1* of *Brassica oleracea* (Konagaya et al. 2008), and silencing of rice *OsCPI* (Lee et al. 2004) and tobacco *NiCPS6* (Zhang et al. 2009) genes in anther lead to male sterility.

Arachis cysteine protease, *AdCP* was identified as one of the differentially expressed genes in the wild peanut, *Arachis diogeni*, when challenged with the late leaf spot pathogen, *Phaeoisariopsis personata* (Kumar and Kirti 2011). Hence, we have made an attempt to investigate the expression of pathogen-induced cysteine protease (*AdCP*) in tapetum for inducing male sterility in transgenic tobacco plants. Male sterile transgenic plants were evaluated based on morphology and fertility analysis. Molecular analysis of transgenic plants was also performed to ascertain the expression of cysteine protease in male sterile plants.

Materials and methods

Plant materials

Tobacco (*Nicotiana tabacum* cv. Samsun) plants grown under green house conditions were used in plant transformation.

Vector construction and development of transgenic tobacco plants

The cDNA of cysteine protease gene (size 1,083 bp, accession no. FJ716616.2) was isolated from a diploid wild species of peanut, *A. diogeni* in a differential expression study by challenging it with the late leaf spot pathogen, *P. personata* to which it was shown to be asymptomatic (Pande et al. 2002, Kumar and Kirti 2011). The full length cDNA obtained by the RACE approach was cloned in pTZ57R vector (MBI Fermentas, Germany) with *XhoI* and *ApaI* restriction sites incorporated in the primers for cloning. P-TA29, a plant gene promoter is a well-characterized promoter from tobacco (Koltunow et al. 1990) that expresses strongly in the tapetum layer of the anther, was used for driving the expression of cysteine protease. TA29 promoter from tobacco genomic DNA and T-NOS terminator from the binary vector were amplified separately and cloned in the pTZ57R vector. Finally, the tapetum-specific expression cassette carrying the TA29 promoter, cysteine protease and the termination signal was excised with specific restriction enzymes and cloned in the binary vector pCAMBIA2300. The confirmed recombinant clones were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (pAL4404) using freeze-thaw method (Chen et al. 1994). Confirmed bacterial strain carrying the recombinant binary vector was used in plant transformation. Tobacco transformation was performed using standard leaf disc transformation procedure (Horsch et al. 1985). The putative, transformed tobacco shoots were selected in three rounds of subculture on shoot elongation medium with 125 mg/L kanamycin. Well-developed shoots were rooted on the rooting medium with the same level of kanamycin in the rooting medium. The regenerated putative transgenic plants were acclimatized at 28 °C in a growth room and then transferred to the green house for further molecular analysis.

Isolation of nucleic acids and semi-quantitative RT-PCR

Total genomic DNA was isolated using cetyl trimethyl ammonium bromide (CTAB) procedure (Doyle and Doyle 1990). Similarly, total RNA from anthers was extracted using TRI-REAGENT method as per manufacturer's instructions (Sigma-Aldrich Corporation, USA). Quality of DNA and RNA was checked on ethidium bromide stained-agarose gels and quantified by standard methods. After DNase-I treatment to total RNA to eliminate the genomic DNA contamination in

RNA samples, first strand cDNA synthesis was carried out by reverse transcription using Superscript-II RT kit (Clontech, USA) as per manufacturer's instruction. Standard methods were used in molecular analyses (Sambrook et al. 1989).

Southern hybridization analysis

Southern hybridization was performed as described in Vijayan et al. (2013). Genomic DNA (10 µg each) from male sterile, semi-sterile and untransformed control plant was completely digested using the restriction enzyme, *HindIII* and the restriction fragments were electrophoresed on 0.8 % agarose gel, and transferred on to Hybond-N⁺ nylon membrane (GE Biosciences, Hong Kong). Following this, the cross-linking of the DNA fragments was carried out using a UV cross-linker (with the energy level at 120 kJ/cm²). The PCR amplified *nptII* marker gene fragment was used as a probe and its radiolabelling was carried out using 'Prime-a-Gene' labeling system (Promega, USA). Southern hybridization was essentially performed as recommended for Hybond-N⁺ nylon membranes by the manufacturer.

Scanning electron microscopy

Pollen grains from both the transgenic as well as non-transgenic control plant anthers at flowering bud stage 7 were placed on adhesive-coated aluminum stubs and coated with gold using sputter coater (Quorum – model Q150RES). For scanning electron microscopy (SEM) observations, pollen grains were photographed by using electrons at an intensity of 10 KV in a Phillips ESEM, model XL30 electron microscope.

Pollen fertility analysis

Pollen grains from three flowers of each transgenic plant were tested in a viability assay using Alexander staining method (Alexander 1969). Pollen germination assay was done using a medium carrying 10 % sucrose and 100 mg/L boric acid and 300 mg/L calcium nitrate (Vijayan et al. 2013), and >500 pollen grains were randomly selected for observation from each plant. Based on viability test and germination assay, plants were classified as fertile, sterile, and semi-sterile.

Histological analysis

Anthers from early developmental stages were fixed in glutaraldehyde, dehydrated in an ethanol series, and embedded in paraffin wax. Sections of 10 µm were cut using a microtome (Leica RM 2125). For microscopic analysis, tissues were stained with toluidine blue and bright field images of anther cross-sections were taken using Olympus UCTR30-2 microscope.

Progeny analysis

The male sterile plants were backcrossed with untransformed fertile wild-type (WT) plant to obtain seeds for T₁ generation. For genetic segregation analysis of kanamycin resistance (*nptII*), the seeds obtained from T₀ plants after crossing with the WT were germinated on a seed germination medium containing 125 mg/L kanamycin to select T₁ transgenic plants carrying the transgenes. After 15 days, germinated seedlings with dark green leaves were scored as positive (carrying the T-DNA) and non-germinated or bleached seedlings as negative. The ratio of positive and negative seedlings was taken for statistical analysis to predict the number of insertions of the T-DNA in the genome of the transgenic plants.

Western blot analysis

The anthers of the male sterile and untransformed control (WT) plants were ground in liquid nitrogen using a mortar and pestle to isolate the total protein. The fine powder was collected in pre-chilled micro-tubes having 1.0 ml of chilled extraction buffer [50 mM Tris-Cl, 1.0 mM β-mercaptoethanol and 1.0 mM PMSF (phenyl methane sulfonyl fluoride)] and incubated at 4 °C for 30 min with intermittent mixing. The tubes were then centrifuged at 4 °C at 15,000 rpm for 20 min and the supernatant containing the protein was collected in a fresh tube. Protein concentration was quantified using the Bradford method with BSA as a standard. Total anther proteins (40 µg) from male sterile and WT plants were mixed with loading buffer, boiled for 5 min and loaded on to 12 % SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a PVDF membrane (Pall Gellmann Corporation, USA). Polyclonal antibody of cysteine protease (generated after the approval of the Institutional Animal Ethics Committee of University of Hyderabad) diluted in the ratio of 1:5,000 was applied followed by ALP conjugated goat anti-rabbit IgG antibody (Bangalore GENEi, India). Recombinant protein expression was visualized after staining with BCIP/NBT (Bangalore GENEi, India).

Results

Characterization of cysteine protease and vector construct preparation

Cysteine protease was identified as one of the genes that have been differentially expressed by a wild peanut, *A. diogeni* challenged with the conidia of the late leaf spot pathogen, *P. personata* (Kumar and Kirti 2011). The cDNA of cysteine protease of *A. diogeni* consisted of a 1,083bp-long nucleotide sequence (NCBI accession no. FJ716616.2), which is potentially capable of coding for a protein of 360 amino acids and

was designated as AdCP. Comparison with other cysteine proteases revealed that (i) AdCP is a pre-pro-protein; (ii) possesses a highly hydrophobic region (amino acid residues 2–18); (iii) Gln-145, Cys-151, His-293 and Asn-320 amino acid residues in the cysteine proteinase active center; and (iv) the highly conserved Gly-Cys-Asn-Gly-Gly motif corresponding to amino acid residues 197–201 (Fig. 1). Along with these features, cysteine residues Cys-147/Cys-198, Cys-182/Cys-231 and Cys-287/Cys-341 are involved in disulfide-bridge formation. It also exhibits a consensus motif $G \times N \times F \times D$ and the non-contiguous ERFNIN signature ($E \times 3R \times 3F \times 3N \times 3I/V \times 3N$) in pro region. It shows significant similarity with soybean Rd19a like cysteine protease (75 %), Brinjal SmCP cysteine protease (72.78 %), *Arabidopsis*, Rd19, (67.78 %), and tobacco (67.5 %).

The cDNA of cysteine protease gene was amplified and cloned in pTZ57R vector. Clones were confirmed through restriction digestion and sequencing. Tapetum-specific promoter (P-TA29, 870 bp) was used in this investigation to drive the expression of cysteine protease gene. TA29 promoter is a well-characterized, dominant, non-leaky, and tapetum-specific promoter with high expression for expressing genes for male sterility. It was amplified from tobacco genomic DNA using TA29 promoter specific forward and reverse primers (Table 1) and cloned in pTZ57R vector. Clones were confirmed through restriction digestion with *KpnI* and *XhoI* enzymes and sequencing. Similarly, T-NOS termination signal from the plasmid DNA of the binary vector was amplified separately and cloned in the pTZ57R vector and the clones were confirmed through restriction digestion.

Subsequently, cysteine protease *AdCP*, TA29 promoter and T-NOS were transcriptionally fused as an expression cassette and cloned in the binary vector pCAMBIA2300 (Fig. 2a). Finally, the construct was mobilized into *Agrobacterium tumefaciens* strain LBA4404 using freeze-thaw method as described in the experimental procedures. The presence of the recombinant binary vector, pCAMBIA2300 was confirmed in bacteria and subsequently used for plant transformation.

Genetic transformation of tobacco with the cysteine protease construct and analysis of transgenic plants

Tobacco transformation was carried out using the standard protocol using leaf discs (Horsch et al. 1985). After cocultivation with *Agrobacterium* cells, the treated explants were transferred on to MS medium supplemented with 2.0 mg/L BAP and 0.1 mg/L NAA, 250 mg/L cefotaxime and 125 mg/L kanamycin for shoot regeneration. Kanamycin-resistant shoots were obtained about a month after initiation of the transformation experiments. Rooting occurred when the shoots were transferred to the growth regulator-free half strength MS medium (rooting medium), and root formation

occurred in about two weeks in the presence of kanamycin. Rooted plantlets were hardened in a mixture of sterile vermiculite and soil (3:1) in a growth room and transferred to the green house for further analysis.

Total DNA was isolated from putative transgenic plants including non-transformed control plant. The presence of T-DNA was confirmed by PCR amplification using the forward and reverse primers for the marker gene *nptII*. PCR amplified 700 bp fragment of *nptII* gene was observed in all transgenic plants and was absent in non-transgenic control plants (Fig. 2b). The *nptII* gene does not carry a *HindIII* site. Hence, the total genomic DNA was isolated from the plants and digested with *HindIII* enzyme. The Southern hybridization for further confirmation of T-DNA integration in the transgenic plants was performed using *nptII* as a probe and the analysis indicated the number of sites of integrations that correspond to the number of bands obtained in the autoradiograph. Different copy numbers were observed in the transgenic plants ranging from single copy to multiple copies (Fig. S1). There were four plants having single copy T-DNA integration in the genome and were designated as 1, 2, 4, and 6.

Total RNA was isolated from anthers of all the plants and semi-quantitative reverse transcription-PCR (RT-PCR) analysis was carried out using *AdCP* specific primers (Fig. 2c). It was observed that complete male sterile plants showed high expression of the cysteine protease transcripts as compared to the semi-sterile plants and there was no expression of cysteine protease transcripts in the anther tissues of the untransformed control plant. Western blot analysis with total protein from anther was performed to ascertain the expression of the transgene encoded protein, the AdCP (Fig. 2d). Complete male sterile plants exhibited increased expression of cysteine protease and no signal was detected in the untransformed control plant.

Tapetal expression of AdCP results in male sterility in high expression plants

From different transformation experiments, 15 independent transgenic plants were generated. During anthesis, their flower morphology and pollen production characteristics were analyzed (Fig. 3). It was found that out of 15 plants, three plants were completely male sterile (transgenic plants numbered 1, 2, and 6) and nine were identified as semi-sterile (transgenic plants numbered 4, 5, 7, 8, 9, 11, 12, 13, 15) in the T_0 generation. Most prominent changes that occurred in the flowers of transgenic male sterile plants included stamen filament and petal size. Stamen filament was reduced by a factor of 1.5 and petal size was decreased by a factor of 1.3 as compared to those in non-transformed wild-type (WT) plants. Similar morphology was also observed in subsequent generations of male sterile plants. Male sterile anthers were small, shrunken, pale green colored, which dried up earlier compared

Brinjal	MDRLFLLS---LLAFALFSSAI-AFSDD-DPLIRQVVS--ETDDNHMLNAEHHFSLFKSK	53
Tobacco	MDRLFLLS---LPRFALFSSAI-AFPDE-DPLIRQVVSETETDDSHLLNAEHHFSLFKSK	55
Peanut	MARLSLL---LLLLVAAVAT---AVDDQADPLIRQVT----DGDHMLNAEHHFTTFKTK	50
Glycine	MANLSILFFGLLLFSAAVATVE-RIDDEDNLLIRQVVP--DAEDHLLNAEHHFSAFKTK	57
Arabidopsis	MDRLKLYFSVFLSFFIVSVSSDVNDGDDLIRQVVG---GAEPQVLTSEDHFSLFKRK	57
Papain	MAMIPSSIS--KLLFVAICLFVYMGLSFGDFSIVGYSQN----DLTSTERLIQLFESWMLK	54
Brinjal	YGKIYASQEEHDFRLKVEKANKLRRARRHQLLDPTAEHGITQFSDLTPSEFRRTYLGLHK-	112
Tobacco	FGKIYASEEEHDFRFKVEKANKLRRARLNQLLDPSAEHGITKFSDLTPSEFRRTYLGLHK-	114
Peanut	FGKSYATQEEHDYRFVFRANLRRAKLHAKLDPSAEHGVTKFSDLTPPEEFKRQYLGLKP-	109
Glycine	FAKTYATQEEHDFRFRIKNNLLRAKSHOKLDPSAVHGVTRFSDLTPSEFRGQFLGLKP-	116
Arabidopsis	FGKVYASNEEHDYRFVVEKANKLRRARRHOKLDPSATHGVTKFSDLTRSEFRKKHLGVRSG	117
Papain	HNKIYKNIDFKIYRFEIEKDNLKYTDETNKKNNSYWLGLNVFADMSNDEFKEKYTGSIAG	114
Brinjal	PRPKLNAQKAPILP--TSDLPEDFDWREKGAVTGVKNQGS CGS CWSFSTTGAVEGAHFLA	170
Tobacco	PKPKVNAEKAPILP--TSDLPADYDWRDHGAVTGVKNQGS CGS CWSFSTTGAVEGAHFLA	172
Peanut	LRLPSTANKAPILP--TSDLPENFDWRDKGAVTPVKNQGS CGS CWSFSTTGAVEGAHFLA	167
Glycine	LRLPSDAQKAPILP--TSDLPTDFDWRDHGAVTGVKNQGS CGS CWSFSAVGALEGAHFLS	174
Arabidopsis	FKLPKDANKAPILP--TENLPEDFDWRDHGAVTPVKNQGS CGS CWSFSATGALEGANFLA	175
Papain	NYTTTELSYYEVLNDGDVNIPEYVDWRQKGAVTPVKNQGS CGS CWSFSAVVTIEGIIKIR	174
Brinjal	TGELVLSLSEQQLVDCDHECDAAEEKSECDAGCNGGLMTTAFEYTLKAGGLQREKDYPYTGR	230
Tobacco	TGELVLSLSEQQLVDCDHECDSEQQDSCDAGCGGGLMTTAFEYTLKAGGLQLEKDYPYTK	232
Peanut	TGELVLSLSEQQLVDCDHVCDPEEYACDAGCNGGLMNNAFDYILQAGGVQTEKDYPSGR	227
Glycine	TGGLVLSLSEQQLVDCDHECDPEERGACDSGCNGGLMTTAFEYTLKAGGLMREEDYPYTGR	234
Arabidopsis	TGKLVLSLSEQQLVDCDHECDPEEADSCDSGCNGGLMNSAFEYTLKAGGLMKEEDYPYTGR	235
Papain	TGNLNEYSEQELLD CDRRS-----YGCNGGYPWSALQLVAQYG-IHYRNTYPYEGV	224
Brinjal	DG-KCHFDKSKIAASVANFSVIGLDEDQIAANLVKHGPLAVGINAA--WMQTYMRGVSCP	287
Tobacco	DG-KCHFDKSKIAAAVTNFSVIGLDEDQIAANLVKHGPLAVGINAA--WMQTYVGGVSCP	289
Peanut	DE-TCKFDKSKVAATVANFSVLSLDEDQIAANLVKHGPLAVGINAI--FMQTYIGGVSCP	284
Glycine	DRGPCCKFDKSKIAASVANFSVLSLDEEQIAANLVKNGPLAVGINAV--FMQTYIGGVSCP	292
Arabidopsis	DGKTCCKLDKSKIVASVSNFSVISIDEEQIAANLVKNGPLAVAINAG--YMQTYIGGVSCP	293
papain	QRYCRSREKGPYAAKTGVRQVQPYNEGALLYSIANQPVSVVLEAAGKDFQLYRGGIFVG	284
Brinjal	LICFKRQDHGVLLLVGYGSAGFAPIRLKEKPYWI IKN SWGENWGEHGYYKICRGH-NICGV	346
Tobacco	LICFKRQDHGVLLLVGYGSHGFAPIRLKEKAYWI IKN SWGENWGEHGYYKICRGH-NICGV	348
Peanut	YICGKNLDHGVLLLVGYGAAGYAPIRFKDKPFWI IKN SWGESWGEDGYKICRGR-NVCGV	343
Glycine	YICGKHLDHGVLLLVGYGSGAYAPIRFKEKPYWI IKN SWGESWGEEGYYKICRGR-NVCGV	351
Arabidopsis	YICTRRLNHGVLLLVGYGAAGYAPARFKEKPYWI IKN SWGETWGENGFYKICKGR-NICGV	352
Papain	-PCGNKVDHAVAAGVYGNP-----YILIKNSWGTGWGENGYIRIKRGTGNSYGV	332
Brinjal	DAMVSTVTATHHTNPNL	363
Tobacco	DAMVSTVTAATHHTNPNL	365
Peanut	DSMVSSVATTFTSSNN	360
Glycine	DSMVSTVAAIHVSNNH--	366
Arabidopsis	DSMVSTVAATVSTTAH-	368
Papain	CGLYTSSFYPVKN----	345

Fig. 1 Comparison of *A. diogeni* cysteine protease (labeled as peanut) with other plant cysteine proteases from Brinjal (SmCP), Tobacco, Glycine (Rd19a like), Arabidopsis (Rd19) and papain

Table 1 Sequences of the oligos/primers used in the present study

Name of the primer	Primer sequence (5′–3′)
TA29 F	GGCGGTACCTTTTGGTTAGCGAATGC
TA29 R	CGGGCTCGAGTTTGTAGCTAATTTCTTTAAG
Cysteine protease F	GCCTCGAGATGGCTCGCCTCTC
Cysteine protease R	TCTAGATTAATTGTTGCTAGATGTAAATG
T-NOS F	GGCTCTAGATAATTCGGGGGATCTG
T-NOS R	CGGAGCTCGTCG ATCGACAAGC
Actin F	TGGCATCACACTTTCTACAA
Actin R	CAACGGAATCTCTCAGCTCC

Recognition sequences for restriction enzyme sites are underlined

to those in fertile flowers, which developed and dehisced normally, releasing fertile pollen.

Pollen viability test was performed using Alexander staining (Fig. 4a, d) and 2 % acetocarmine staining (Fig. S2). It revealed that fertile pollen grains have stained positively with uniform dark coloration, while pollen grains of the sterile plants did not get stained. Furthermore, SEM observations also revealed that the pollen of male sterile line were shrunken, deformed and with exine sculpturing appearing smooth, spherical to elliptical, irregular-shaped grooves with fossulate orientation, when compared to the normal control pollen having regular perforated orientation over the exine (Fig. 4b, c, e, f).

The germination frequency of pollen grains was observed on sucrose-boric acid medium (Fig. 5). Under normal conditions, fertile pollen grains started germinating within 2–3 h.

Pollen grains from untransformed control fertile plants exhibited 60–70 % of germination, while semi-sterile plants showed decreased pollen germination ability. Pollen grains from completely sterile plants failed to germinate even after 24 h also (Table 2). Similar observations were made in male sterile lines in subsequent generations also.

Fertility analysis

Fertility in the transgenic plants was also determined by observing the capsule formation efficiency after selfing or backcrossing with pollen from non-transformed WT plants (Fig. S3). Male sterile flowers were unable to form capsules after selfing. However, manual pollination of male sterile flowers with the pollen from untransformed control plants resulted in normal capsule and seed formation.

Histological study of the anther

A histological study was performed by taking the transverse sections of the developing anthers from the male sterile and non-transformed control plants (Fig. 6). Study of anther sections showed that anther sacs of the flowers in fertile plants were filled with normal round-shaped pollen grains, while anther sacs of male sterile flowers exhibited very few and deformed pollen grains. Also, the tapetal layer in the anthers of the sterile plants displayed ablated nature and appeared to be unsuitable for supporting the development in the microsporogenous tissues in contrast to the tapetal layer in

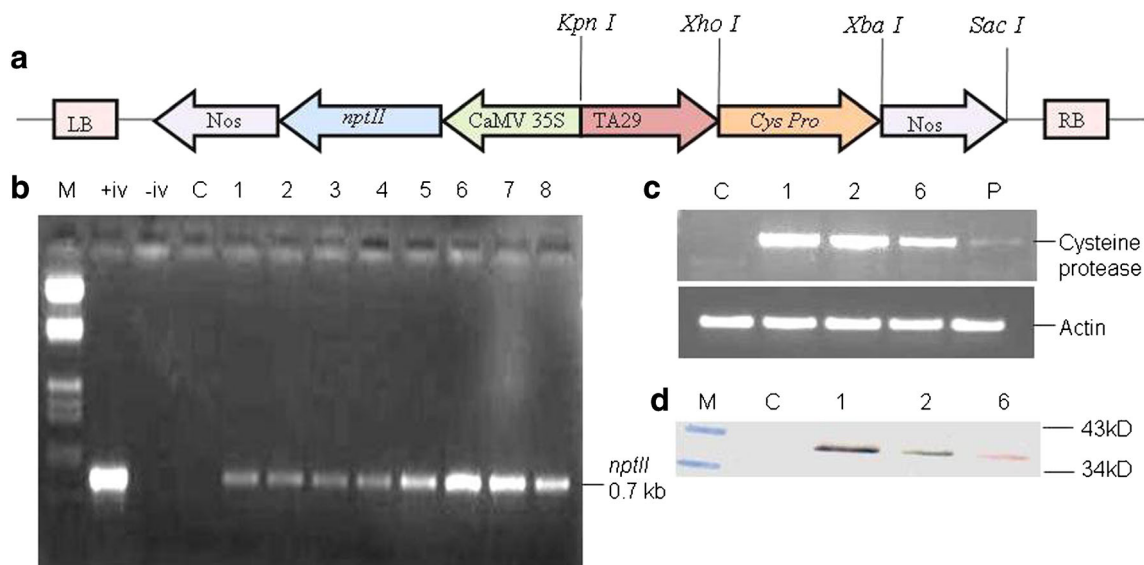


Fig. 2 Molecular analysis of T₀ TA29-Cysteine protease transgenic tobacco plants. **a** Schematic representation of cysteine protease construct. **b** PCR confirmation of TA29-Cysteine protease transgenic plants using *nptII* forward and reverse primers that would amplify a 0.7-kb fragment. **c** Semi-quantitative RT-PCR analysis of cysteine protease expression in the anther of untransformed control (C), partial male sterile (P) and complete

male sterile transgenic plants (1, 2, 6). cDNA was synthesized from total RNA from anther of control and male sterile transgenic plants and amplified with cysteine protease specific forward and reverse primer. Actin served as an internal control. **d** Western Blot analysis of transgene encoded protein AdCP. Cysteine protease expression analysis in the anther of male sterile lines (1, 2, 6) and untransformed control plant

Fig. 3 Comparison of flower morphology of male sterile T₂ transgenic plants and fully opened non-transformed control plant flower (a, c). Fully opened, flower of non-transformed control plant (b, d). Stamen length has been reduced in male sterile transgenic plants (e) compared to the non-transformed control plant (f)



Fig. 4 Pollen characteristics of male sterile transformed and untransformed control plants. a and d are Alexander stain images, b, c, e, and f are SEM images. a, b, c Untransformed control plant pollen, d, e, f sterile pollen. Scale bar 25 μm

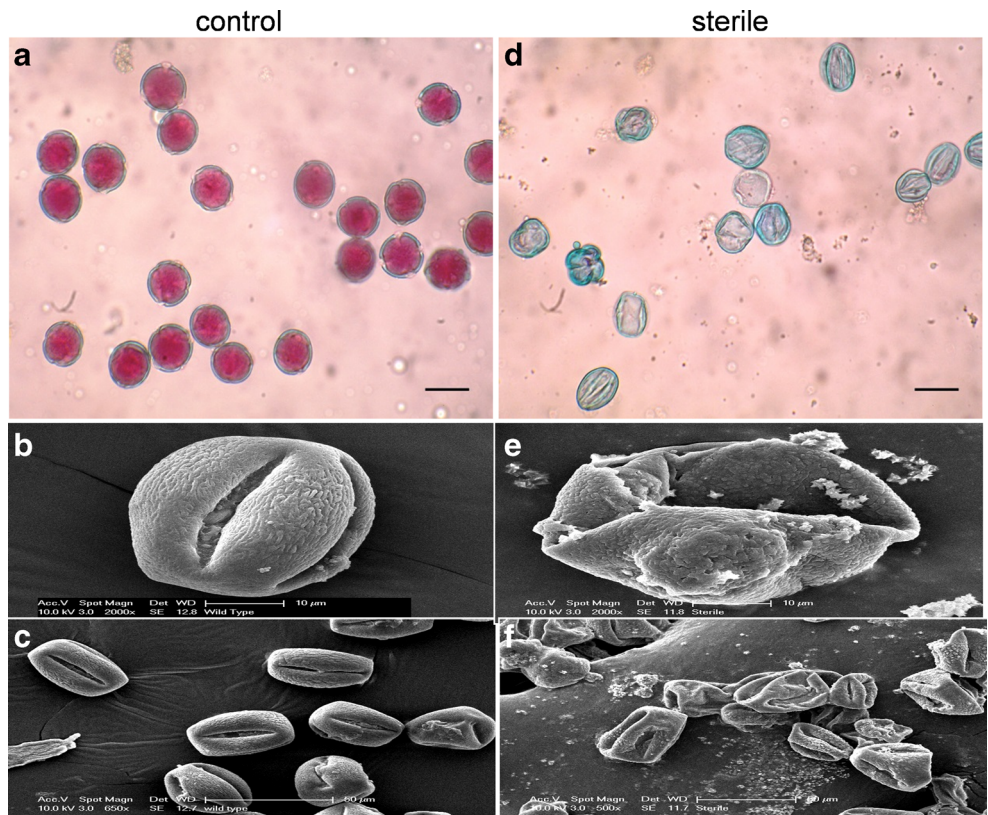
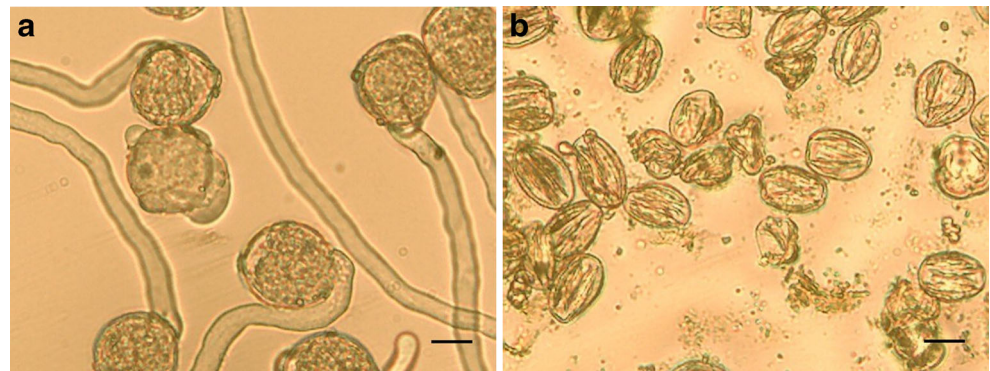


Fig. 5 In vitro pollen germination of untransformed control plant and sterile transgenic plants. Pollen grains were germinated on sucrose-boric acid medium and >500 pollen grains were observed. **a** Untransformed control plant pollen, **b** Sterile pollen. Scale bar 25 μ m



the anthers of the fertile flowers, which developed normally and appeared conspicuous to support the growth of the microsporogenous tissue.

Segregation analysis

Segregation analysis was performed with the three male sterile transgenic plants (designated as 1, 2, and 6) after controlled pollination with pollen from the wild-type plants. The selection for the segregants carrying the transgenes contained 125 mg/L kanamycin in the seed germination medium, which completely arrested the germination of seed from plants that do not carry and express the *ntpIII* gene. Hence, the segregation into plants carrying the transgene and nulls can be observed. Seed germination of T₁ transgenic progeny of the primary male sterile transgenic plants showed 1:1 segregation ratio (Table 3) with respect to kanamycin resistance clearly showing that they are transgenic plants with single copy insertions.

Table 2 Evaluation of pollen viability and in vitro pollen germination rate in *AdCP* expressing transgenic (T₀) plants

Transgenic line (T ₀)	Pollen viability (%)	Germination frequency (%)
1	0	0
2	0	0
3	92~98	58~66
4	15~19	4~6
5	53~59	34~40
6	0	0
7	11~15	7~11
8	62~72	29~37
9	38~46	25~31
10	82~90	54~60
11	8~14	5~9
12	20~26	10~16
13	24~32	14~20
14	88~98	68~76
15	36~42	22~26

Greater than 500 pollen grains were randomly selected for observation from each plant

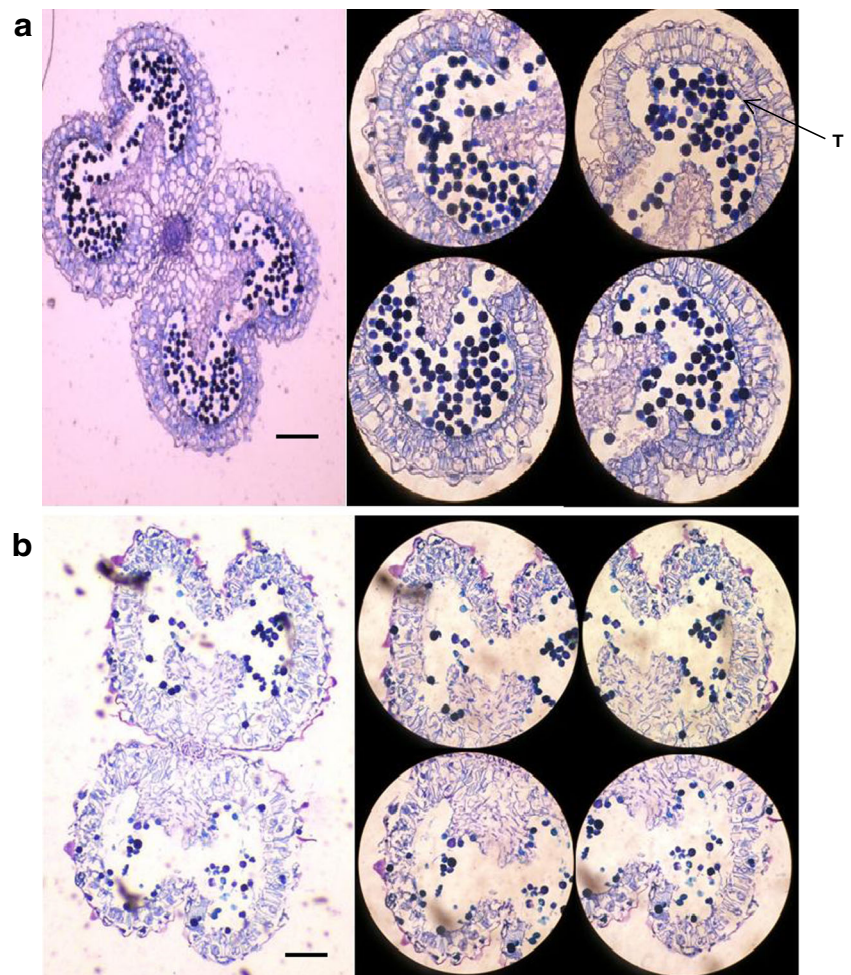
Discussion

Male sterility is the simplest technique to develop hybrid varieties in various crop plants for stability of performance over years and environments. However, usable male sterility systems have not yet been generated through conventional breeding methodologies for several important crop plants. In such a scenario, genetic engineering becomes an efficient and rapid approach for developing male sterile lines in them. Most of these genetic manipulation-based approaches are based on early ablation/destruction of tapetal cell lineages in the anthers of the flowers, which provide nourishment to the developing microsporogenous tissues from which fertile pollen are generated. In general, tapetal cells die after nourishment during male gametophyte development. The timing of tapetal cell death is important for maturation of pollen grains. Any interference with the tapetal cell development results in sterility of the pollen grains.

In the present study, a pathogen-induced cysteine protease (*AdCP*) from *A. diogeni* was used for developing male sterile tobacco plants. Cysteine protease is an endopeptidase enzyme belonging to the papain family of proteases. Cysteine protease emerges as a key regulator of animal programmed cell death. Many reports suggest their involvement in many developmental events of plant programmed cell death (Wan et al. 2002; Solomon et al. 1999). *SEN11* and *SEN102*, papain-like cysteine proteases have been shown to be expressed during flower senescence in *Hemerocallis* spp. (daylily) (Guerrero et al., 1998) and a similar gene, *PRT5* is expressed during tepal senescence in *Sandersonia aurantiaca* (Eason et al., 2002). The expression of eggplant (*Solanum melongena*) *SmCP* is associated with programmed cell death during leaf senescence, fruit senescence, xylogenesis and anther senescence (Xu and Chye, 1999; Xu et al., 2003). *AdCP* also showed significant similarity with *S. melongena* cysteine protease (*SmCP*, 72.78 %). This suggests that the present cysteine protease also might have a role in programmed cell death and could be deployed for inducing male sterility.

Early destruction of tapetal cell lineage in anther has been widely used strategy for this purpose as detailed out earlier

Fig. 6 Transverse section of anther of untransformed control plant (a) and transgenic male sterile plant in T₂ generation (b). Observe the copious presence of fertile pollen in the anther sacs of the fertile flower with proper tapetal cell layer, while the sterile anther shows reduced number of pollen grains that are sterile with the ablation of tapetum (T). Scale bar 50 μm



including Mariani et al. (1990) and Nizampatnam et al. (2009). The tapetum-specific promoter without leaky expression (TA29) from tobacco that has been widely used in undertaking the ablation of tapetal tissue in earlier cases has been used in the present investigation also to drive the expression of pathogen-induced cysteine protease. TA29 promoter has been shown to express during early stages of tapetum development and was efficiently used for the expression of gene for tapetal cell death.

TA29-AdCP construct was developed in binary vector pCAMBIA2300 and transformed into tobacco using *Agrobacterium* strain LBA4404. Stable T-DNA integration

Table 3 Segregation analysis of male sterile T₁ seeds by germination on selection medium containing 125 mg/L kanamycin

Male sterile T ₀ plants	No. of seeds germinated on selection medium	χ^2	Probability
1	117/226	0.283	0.5947
2	68/132	0.12	0.7290
6	82/170	0.211	0.6459

in the PCR positive plants was confirmed and confirmed transgenic plants were analyzed for their flower morphology and pollen production characteristics. Three transgenic plants showed distinct flower morphology that is reduction in the length of stamen filament, which has been correlated with the arrest of microsporogenesis (Mariani et al. 1990). SEM study and histological analysis further confirmed the sterility phenomenon as described earlier. Female fertility did not get affected as capsule formation was normal when male sterile plants were pollinated with the pollen grains from untransformed control plants. This indicates that tapetal expression of cysteine protease affects only male gametophyte development, not the female gametophyte development and the related female fertility. These observations indicate that AdCP expression in tapetum resulted in improper microsporogenesis of transgenic tobacco plants causing male sterility. RT-PCR analysis and western blot results also demonstrated correlation of cysteine protease expression and male sterility of the plants.

Our results are analogous to the report on *BoCysP1* (Konagaya et al. 2008) where *Brassica* cysteine protease (*BoCysP1*) was shown to be involved in PCD of inner integument (Wan et al. 2002). Overexpression of *BoCysP* gene,

driven by A9 and A3 promoters separately, was used to induce male sterility in *Arabidopsis*. In contrast to this, silencing of rice *OsCPI* (Lee et al. 2004) and tobacco *NtCPI56* (Zhang et al. 2009) also resulted in the sterility of the pollen grains. It was explained as the lethality resulting from the down regulation of the corresponding cysteine protease with a specific function in fertile pollen generation as an increase in protease activity during anther development (DeGuzman and Riggs 2000). Similarly, Bnms3 mutant of *Brassica napus* displays abnormal tapetal degradation by modulating the expression of cysteine protease genes (Zhou et al. 2012) and proteome analysis of YX-1 male sterile mutant of wolfberry (*Lycium barbarum* L.) anther also showed differential expression of several proteins including down regulation of cysteine protease (Zheng et al. 2012). These observations suggest that disturbances in the protease cascade prior to the tapetal cell PCD could lead to pollen abortion and male sterility. Thus, it can be speculated that AdCP-induced male sterility is the result of disturbance in the protease cascade prior to tapetal PCD. Detailed investigation is required to establish the link between AdCP and PCD.

In conclusion, using *AdCP* as a plant gene for inducing male sterile crop plants would be a good approach in modern biotechnology. To some extent, it will address environmental and consumer safety-related concerns in using GM crops as the protein is a regular plant-based protein with defined roles in plant development. The present system of generating male sterility can be directly used in vegetable crop plants in which vegetative parts of the plant form the economic produce.

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